STANDARD OPERATING PROCEDURE

General Use of a Microtiter Plate ELISA Kit for the Determination of Diazinon in Water

KEY WORDS

Enzyme-Linked Immunosorbent Assay, microtiter plate, diazinon

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1.0 INTRODUCTION

An Enzyme-Linked Immunosorbent Assay (ELISA) is a physical assay, characterized by the Law of Mass Action, which relies on the reaction of a target analyte (or antigen, Ag) with a selective antibody (Ab) to give a product (AbAg) that can be measured:

$$Ab + Ag \underset{K_{-1}}{\overset{K_{1}}{\rightleftharpoons}} AbAg$$

In a competitive, magnetic particle-based ELISA, the antibody is bound to particles that are suspended in solution. When a test sample containing an antigen and an antigen-enzyme conjugate are added, competitive inhibition occurs between the antigen-enzyme conjugate and an unlabeled antigen. After the formation of an immune complex from an antigen-antibody binding, the reagents are separated by a washing, after which a color substrate is added to the immune complex. If the antigen-enzyme conjugate is in excess, a color change will occur. If the unlabeled antigen is in excess, there will be little to no change in color. Color development is inversely proportional to the diazinon concentration, such that darker color signifies lower concentration and lighter color is indicative of higher concentration.

ELISA has become an important and cost effective alternative data gathering and screening method for the determination of pesticides in environmental matrices, particularly for the analysis of large numbers of samples. For routine testing or screening of pesticides, for example, ELISA analysis may cost as much as 75% less than comparable GC/HPLC analysis. The California Department of Pesticide Regulation (CDPR) routinely uses commercial ELISA test kits as a screening or data gathering method for monitoring pesticide residues in compliance monitoring as well as research studies. This SOP describes the procedures for the general use of a commercial microtiter plate ELISA kit, and outlines specific performance assessment guidelines, including a summary of the materials, methods, calculations, and statistical procedures necessary to quantitatively measure and evaluate method detection limit, method accuracy, and method precision.

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1.1 Purpose

To provide standardized instruction for the use of microtiter-based ELISA kits.

1.2 Definitions

1.2.1 **Competitive Enzyme-Linked Immunosorbent Assay (ELISA):** an immunoassay format in which the target analyte contained in an aliquot of a sample competes with an enzyme-labeled analyte (conjugate) to bind with an immobilized analyte-specific antibody (e.g., antibody adsorbed to a plastic plate or to magnetic particles). The amount of sample analyte present is indicated by the amount of color developed when a color solution is added to the assay. Lighter color is associated with lower levels of enzyme conjugate binding with the antibody due to higher levels of sample analyte that bind with the antibody.

1.2.2 **Immunoassay:** a biochemical test that measures the level of a substance (analyte) in a liquid, using the reaction of an antibody or antibodies to its antigen.

1.2.3 **Antigen:** a substance that can elicit the formation of antibodies and react specifically with the antibodies produced.

1.2.4 **Antibody:** a protein that selectively recognizes and binds to a target analyte or group of structurally related analytes.

1.2.5 **Hapten:** Pesticides and other small molecules are not themselves large enough to elicit an antigenic response, but when conjugated to a large molecule (such as a protein) they can stimulate the formation of antibodies. Conjugation is accomplished by adding functional groups to the target analyte that will facilitate its binding to a carrier protein. These groups, called spacer arms, contain functional groups such as -OH, -COOH, -SH, or -NH_2. The combination of a target analyte with a spacer arm is called a hapten.

1.2.6 **Conjugate:** the coupling of a hapten to a carrier protein, such as an enzyme.

1.2.7 **Cross-reactivity:** the ability of compounds that are structurally related to the target molecule to bind to the target-specific antibody.

1.2.8 **Matrix effects:** non-specific reactions (interferences) between one or more ELISA components (e.g., antibody, conjugate, target analyte) and constituents of the substance containing the target analyte (e.g., surface water, soil or plant extract), which may lead to inferior test results.

1.2.9 **Negative control:** A control sample that is known to be devoid of the antigen of interest. For the analysis of the Strategic Diagnostics ELISA kits, the negative control is DI (distilled, deionized) water.

1.2.10 **Accuracy:** the proximity of a measured value to the "true" value.

1.2.11 **Precision:** the proximity of a value to the mean of a series of measured values obtained from repeated measurement of the same sample, without reference to its agreement with the "true" value.

1.2.12 **Dose-response curve-** Representation of the signal generated by an immunoassay (y axis) plotted against the concentration of the target compound (x axis) in a series of standards of known concentration. When plotting a competitive immunoassay in a rectilinear format, the dose-response will have a hyperbolic character. When the log of concentration is used, the plot assumes a sigmoidal shape, and when the log of signal is plotted against the *logit* transformation of concentration, a straight-line plot is produced.

1.2.13 **Optical density (OD)-** Synonymous with absorbance, Optical Density is the amount of light being absorbed at a given frequency, as given by the following equation:

$$OD = \log I_0 - \log I_T$$

where

I_0 is the intensity of the incident light

I_T is the intensity of the transmitted light

1.2.14 **Target analyte-** the compound for which the ELISA has been developed.

2.0 MATERIALS

2.1 Diazinon EnviroGard¨ ELISA kit (Strategic Diagnostics, Inc., Newark, N.J.):

a). Diazinon antibody (rabbit polyclonal anti-diazinon) coated strips (8 strips, 12 wells each).

b). Strip Holder.

c). Diazinon horseradish peroxidase (HRP) labeled enzyme conjugate.

d). Color solution (hydrogen peroxide and 3,3',5,5'-tetra-methylbenzidine).

e). Stopping solution (0.5% sulfuric acid).

f). Washing solution (non-buffered deionized water).

g). 1 vial of stock solution (100 μgL^{-1} diazinon in methanol).

2.2 Dynatech microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) for serial dilutions

2.3 Parafilm flexible, self-sealing film

2.4 Dual wavelength (set at 450-650 nm) VMax microplate reader and SoftMax Pro microplate data acquisition software (Molecular Devices, Menlo Park, CA). A computer (PC or Mac) is required to run the software (for system requirements, see http://www.moleculardevices.com/downloads/SMP5xSystemReqs.pdf)

2.5 Eppendorf Series 2000 adjustable-volume (100-1000 μL) Reference sampling pipette (Eppendorf, Hamburg, Germany)

2.6 Eppendorf Titermate 12-channel adjustable-volume (100-300 μL) sampling pipette to dispense liquids

3.0 PROCEDURES

3.1 Calibrator preparation (aqueous calibrators are unstable and should be prepared fresh prior to each new analysis)

3.1.1 Allow the 100 μgL^{-1} (ppb) stock solution of diazinon to come to room temperature. Swirl vial before pipetting.

3.1.2 Prepare the 500 ngL^{-1} (ppt) calibrator by pipetting 50μL of the 100 ppb diazinon stock solution into a 10 ml volumetric. Bring to volume with distilled, deionized (DI) water.

3.1.3 Prepare the 100 ngL^{-1} (ppt) calibrator by mixing 1.0 ml of the 500 ngL^{-1} calibrator with 4.0 ml DI water.

3.1.4 Prepare the 30 ngL^{-1} (ppt) calibrator by mixing 0.30 ml of the 500 ngL^{-1} calibrator with 4.7 ml DI water.

3.1.5 Use DI water as the negative control.

3.2 Assay procedure

3.2.1 The raised markings on the strip holder identify the well location while the reagents and samples are added. To add the calibrators, samples, conjugate, substrate, and stop solution, a 100 mL pipette must be used.

3.2.2 Two strips may be used to run the negative control (NC), three calibrators (C), and eight samples (S) in duplicate, as illustrated below. More strips may be added to accommodate additional samples as needed. Add 100μL of the negative control and calibrators to their respective plate wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	C	C	C1	C1	C2	C2	C3	C3	S1	S1	S2	S2
B	S3	S3	S4	S4	S5	S5	S6	S6	S7	S7	S8	S8
C												
D												
E												
F												
G												
H												

3.2.3 Add 100μL of the samples to be analyzed to their respective plate wells

3.2.4 Using the same order of addition as above, add 100μL of diazinon-enzyme conjugate to each well and gently mix the contents of the wells by moving the plate in a circular motion on the bench top for 1 minute.

3.2.5 Cover the plate with acetate sealing tape or parafilm to minimize evaporation and allow to incubate at ambient temperature for 1 hour.

3.2.6 After the incubation period, remove tape and shake out the contents of the wells into a sink.

3.2.7 Wash wells 6 times with DI water and tap dry.

3.2.8 Add 100μL of color substrate (Is this the same as the color solution on the materials list? If so, use that term here or change the term in the materials list) to each well and gently mix the contents of the

wells by moving the plate in a circular motion on the bench top for 1 minute.

3.2.9 Cover the plate with acetate sealing tape or parafilm (Add to list of materials) to minimize evaporation and allow to incubate at ambient temperature for 30 minutes.

3.2.10 After the incubation period, remove tape and add 100μL of stopping solution to each well.

3.3 Reading plate results on a Vmax microtiter plate reader

3.3.1 Adjust the wavelength of the microtiter plate reader to 450 nanometers (nm). If it has dual wavelength capability, use 600 or 650 nm as the "reference" wavelength.

3.3.2 If the plate reader does not auto-zero on air, zero the instrument against 120μL water in a blank well, then measure and record the optical density (OD) of each well. Or, measure and record the OD in every well, then subtract the OD of the water blank from each reading.

3.3.3 Use a semi-log curve fit for the standard curve.

3.4 SoftMax software setup procedures

3.4.1 Follow the instructions in the documentation materials that came with the software. Instructions vary depending upon the type of computer (PC or Mac) that is interfaced with the microtiter plate reader, the operating system version installed on the computer, and the particular version of SoftMax software installed.

3.5 Plate loading and plate reading procedures

3.5.1 Turn on the microtiter plate reader using the switch on the right rear of the unit. The plate microtiter plate holder will open automatically.

3.5.2 Place the microtiter plate in the holder and press the yellow key and the drawer key to close.

3.5.3 On the instrument, choose "control," followed by "read plate". The plate will be read in a few seconds and results can be viewed using the "display", "raw data", "plate", and/or "report" menu options. The curve can also be displayed by choosing the "display", "analyzed", "curve", and/or "report" menu options.

3.5.4 To print the results, choose "print window" command from the "file" menu option.

4.0 CALCULATIONS

4.1 Spectrophotometric measurement and analysis

4.1.1 From measured optical densities (OD), calculate the mean OD value for each of the standards and samples

4.1.2 Calculate $\%B_O$ for each of the calibrators/unknowns:

$$\%Bo = (\text{average OD of calibrators or unknowns})/(\text{average OD of negative control}) * 100$$

4.1.3 Construct a standard curve by plotting the %Bo for each standard on vertical linear (Y) axis versus the corresponding diazinon concentration (C) on a horizontal log (X) axis:

$$\%B_O = m(\log C) + b \qquad (1)$$

where

m = slope
b = intercept
C = concentration of diazinon in unknown

The calibration curve should have a correlation coefficient r > 0.99 (R2>0.98). If not, repeat the measurements. If replicate

measurements fail, it will be necessary to repeat the preparation and analysis of calibrators per Section 3.1 – 3.5 above.

4.1.4 %Bo for controls and samples will then yield levels in ppb of diazinon by direct calculation solving the resulting equation of the line for log C:

$$C_{diazinon} = 10^{(\%B_o - b)/m}$$

4.1.5 The manufacturer (Strategic Diagnostics, Inc., Newark, N.J.) defines the limit of detection (LOD) as 85% Bo (where Bo is the average optical density (OD) of the calibrator or sample). For the determination of the LOD on an assay to assay basis, subtract three times the standard deviation (SD) of the negative control from the its mean optical density (MOD)

$$LOD\ (\%B_0) = MOD_{negative\ control} - 3*SD_{negative\ control} \qquad (4)$$

which gives the LOD in terms of $\%B_0$. Substitute LOD ($\%B_0$) into the equation of the line generated above to convert to units of concentration. Alternatively, the minimum concentration of substance that can be measured may be expressed in terms of the method detection limit (MDL). The MDL is defined as the minimum concentration of substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. The MDL is determined by multiplying the appropriate one-tailed 99% t-statistic by the standard deviation (σ) obtained from a minimum of three replicates (seven recommended) of a matrix spike subsample containing the analyte of interest at a concentration one to five times the estimated MDL:

$$MDL = t_{(n-1,\ \alpha = 0.99)}\ (\text{standard deviation}) \qquad (5)$$

The t-statistic is obtained from standard reference tables or from the table below.

Number of Samples:	*t-statistic*
3	6.96
4	4.54
5	3.75
6	3.36
7	3.14
8	3.00
9	2.90
10	2.82

4.2 Quality Control

The following QC indicators should be reported with ELISA results each time an analysis is performed. All blanks, calibrators, and unknowns should be run in replicate for every assay. At the minimum, samples should be analyzed in duplicate. For better quality QC results, increase the number of sample and or QC replicates per the needs expressed in the particular study design.

4.2.1 Accuracy

Measure calibrators (minimum duplicate samples), calculate the estimated concentration of each unknown using Equation (3), and express as the percent recovery (%R) as the mean estimated (observed) concentrations relative to expected (known) concentrations:

$$\text{Accuracy} = \%R = (C_{observed} / C_{expected}) * 100$$

Acceptable recoveries fall within the range 70 – 120%.

4.2.2 Precision

Precision is determined from the repeated measurement of the same control sample. To obtain this parameter, repeat the spectrophotometric measurement of the 1.8 ppb positive control supplied in the kit at least 4 times. Precision may then be expressed as the relative standard deviation (RSD) or the percent coefficient of variation (%CV):

RSD = %CV = (standard deviation of control/mean conc. of control [ppb]) * 100

Values of RSD/%CV should not exceed ± 20%.

4.3 Matrix Effects

Typically, interferences are quantified by comparing a standard curve produced in a control matrix such as distilled or buffered water with a calibration curve generated in the matrix of interest. The ensuing relative slope of a standard calibration curve in a matrix containing interferences is less steep than with the control system. Thus,

$$m_{\text{matrix system}} < m_{\text{control system}}$$

for those matrices having interfering components. An alternative method for quantitatively assessing matrix interferences is also available. Absorbance values for matrix blanks are first normalized with respect to the absorbance of the blank control matrix,

$$I_m = [ABS_{\text{Blank A}} - ABS_{\text{Blank B}}] / ABS_{\text{Blank A}}$$

where ABS is the mean absorbance determined from experiment, A is the control matrix (e.g., DI or buffered water), and B is the unspiked environmental matrix. The term I_m is known as the *index of matrix interference*. Upon calculating I_m for a particular matrix, it is then used to derive a correction factor, N

$$N = [(100-I_m)/100]$$

which is subsequently employed for the direct quantitation of the analyte of interest

$$C_X = NC_{measured}$$

where C_X is the actual analyte concentration and $C_{measured}$ is the analyte concentration determined from the calibration curve. With this approach, the calculated I_m values can be considered a "true" matrix interference, thus allowing the determination of the analyte in each matrix directly from the calibration curve in the control matrix.

4.4 Bias

The situation often arises that a kit can react with far more substances that can be measured by full protocol methods, thus generating positive bias for ELISA. To account for potential bias due to matrix effects, measured values are compared to an accepted reference value in a sample of known concentration or by determining the recovery of a known amount of contaminant spiked into a sample, i.e., a matrix spike. Bias due to matrix effects based on a matrix spike is calculated as

$$Bias = (x_S - x_u) – K$$

where

x_S = measured value of spiked sample

x_u = measured value of unspiked sample

K = known value of spike in the sample

The percent recovery (%R) is then determined from the following equation:

$$\%R = [(x_S - x_u)/K]*100$$

5.0 REPORTING REQUIREMENTS

Samples yielding measured concentrations less than the LOD are reported as *nd* or "none detected." Samples which yield concentrations greater than the LOD but less than the linear range of the kit (0.022 ppb) are reported as "< 0.022 ppb." If samples yield concentrations greater than the linear range of the kit (0.500 ppb), they are reported as "> 0.500 ppb."

6.0 REFERENCES

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